

Rotavirus and other viral diarrhoeas*

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Recent evidence indicates that viruses are an important cause of acute diarrhoea in infants and young children in both developed and developing countries. This article reviews the available information on the epidemiology, clinical features, and laboratory diagnosis of acute diarrhoea due to two of the more important and recently discovered viruses, namely rotaviruses and the Norwalk and Norwalk-like agents, or to other viral agents. Research priorities are also recommended that will help to elucidate the epidemiology, pathophysiology, and means of preventing viral diarrhoeas. Foremost among these research priorities is the development of a rotavirus vaccine for use in man.

The problem of viral diarrhoea is a particularly important topic since recent evidence has indicated that viruses are responsible for the majority of diarrhoeal episodes in infants and young children in both developed and developing countries and may account to a considerable extent for malnutrition owing to associated malabsorption. Identification and characterization of these viruses and a clear understanding of their epidemiology are necessary in order to be able to prevent their transmission and, especially, to develop suitable vaccines.

ROTAVIRUS DIARRHOEA

Rotavirus diarrhoea in man

The virus

Rotavirus was first detected in man in Australia in 1973 by thin-section electron microscopic examination of duodenal biopsies obtained from children with acute diarrhoea, and soon afterwards in Australia, Canada, the United Kingdom, and the United States of America by electron microscopic examination of diarrhoeal stool specimens. The virus is 70 nm in size, contains RNA, and has an inner and outer capsid. It derives its name from the Latin word "rota", meaning wheel, which it resembles in appearance. Initially, it was also referred to as "orbivirus", "duovirus", "reovirus-like agent", and "infantile gastroenteritis virus".

Human rotaviruses are morphologically similar and share certain common antigens with animal rotaviruses. By complement fixation (CF) the human rotavirus has been shown to be closely related to at least 4 animal rotaviruses: Nebraska Calf Diarrhoea Virus (NCDV), Epizootic Diarrhoea of Infant Mouse virus (EDIM), Simian Agent (SA)-11, and the offal (O) agent of sheep and calves. In fact, these four animal antigens can be used as CF antigens for detecting serological evidence of rotavirus infection in man; this has been useful for epidemiological studies since the supply of human rotavirus is limited because it has not been propagated efficiently in cell culture. (The relationship between animal and human rotaviruses is discussed further on pages 186-188.)

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¹ This article is based on the Report of a Sub-group of the Scientific Working Group on Epidemiology and Etiology, CDD Programme, held in Washington, DC, in March 1979. The participants in the Sub-group are listed on page 198. A French translation will be published in a future edition of the *Bulletin*.

Clinical features

The incubation period of rotavirus enteritis ranges from 1 to 7 days, but is usually less than 48 hours. In one volunteer study the onset of diarrhoea occurred 2–4 days after oral administration of the rotavirus-containing inoculum. In the usual case of rotavirus enteritis, vomiting is a prominent early symptom and in many cases precedes the onset of watery diarrhoea. Mucus is found in the stool in up to 25% of cases but blood is rare. Mild temperature elevation occurs in about 30–50% of cases. Respiratory symptoms have been reported but there has been no evidence to indicate that they were caused by the virus. As in other enteric infections, the severity of symptoms varies. The average duration of illness is 5–7 days and virus shedding continues not infrequently for up to 10 days. In the more severe cases seen at treatment centres, severe dehydration and electrolyte imbalance have been observed. Deaths from rotavirus diarrhoea have been reported, although the impact of rotavirus disease on infant mortality in developing countries has yet to be determined.

In adults infected with rotavirus, mild diarrhoea or, more commonly, subclinical infection occurs, probably because of active immunity (see page 185). For reasons that are unknown, newborns infected with rotavirus are also often asymptomatic or have only mild disease.

Treatment of rotavirus disease generally requires standard rehydration therapy. This can be administered orally in all but the most severely dehydrated patients who are unable to drink, or in patients with intractable vomiting. The glucose–electrolyte oral rehydration solution (ORS) developed originally for the treatment of cholera has been used successfully for the rehydration of rotavirus diarrhoea cases in a number of developing countries.^a

An understanding of the pathophysiology of rotavirus diarrhoea has been derived from a sequential biopsy study of gnotobiotic colostrum-deprived calves challenged with human rotavirus. This study revealed a sequence of events in the small intestine consisting of infection of the absorptive villous epithelial cells, replacement of the tall columnar villous epithelial cells with cuboidal cells, shortening of the villi, lymphocytic infiltration of the villous lamina propria, and repair. Such changes appeared in a cephalocaudal direction and suggest that much of the diarrhoea may be related to a loss of absorptive capacity in the small intestine. Similar morphological findings were observed in small intestinal biopsy studies in infants.

Human rotavirus can also induce diarrhoeal illness in other newborn, colostrum-deprived animals, including gnotobiotic and conventional piglets, rhesus monkeys, and gnotobiotic lambs. It can also infect but not induce illness in newborn puppies. This finding that only newborn animals are susceptible to human rotavirus has not been explained. One hypothesis is that this predilection may be the result of higher lactase concentrations in the infant animal, the lactase acting as a receptor and an uncoating enzyme for the rotavirus.

A question of considerable interest is whether rotavirus could be responsible for cases of chronic diarrhoea, but no studies have been done to test this hypothesis. Patients with acute rotavirus diarrhoea usually have increased reducing substances in their stool, reflecting defects of absorption and of digestion of carbohydrates. This is not surprising given the pathophysiology of the disease, but these abnormalities do not prevent hydration with glucose–electrolyte oral solutions.

Incidence

Rotavirus enteritis is generally a disease of infants and young children and appears to have a worldwide distribution. It has been the most frequently observed virus in the stools of infants and young children with diarrhoea in almost all areas where it has been looked

^a Report of the Scientific Working Group on Clinical Management of Acute Diarrhoea, 1978, unpublished document WHO/DDC/79.3.

for. Most cases are children 6–24 months old with a peak incidence at 9–12 months. In a number of hospital-based studies carried out in infants and young children in developed and developing countries, rotavirus has been detected in approximately 50% of diarrhoea cases, sometimes with seasonal variation (see below). In some studies the number of male cases has been up to 20% higher than that of female cases, but whether this is due to a greater susceptibility or exposure of male children, or to a higher likelihood of their being brought for medical care is not known. Data from community-based studies are much more limited, but one carried out in Guatemala and one in Bangladesh suggest that rotavirus accounts for approximately 10–20% of all community diarrhoea cases.

A number of serosurveys have been made to determine the frequency of rotavirus infection in early life. In a study of persons living in the Washington, DC, area, rotavirus antibody was detected by CF and immunofluorescence in over 90% of children by the third year of life. In Melbourne, Australia, 40% of infants 2 months of age or less possessed rotavirus CF antibody; by 3–5 months of age the percentage with antibody had increased and approached 70% by the age of 3 years. Approximately 70% of adults were also shown to have antibody. Workers in Toronto have reported similar results. In a serosurvey done in Vellore, India, in which rotavirus antibody was measured by counter-immunoelectrophoresis, 85% of newborns were shown to have antibody; by the fifth month 30% and by the third year of life nearly 90% had evidence of prior infection. Using the more sensitive enzyme-linked immunosorbent assay (ELISA), rotavirus antibody prevalence was found to be 100% in European and Aboriginal populations 2–60 years of age living in the same area in Australia and in adults and children living in an area of southern India. Other surveys using a variety of serological techniques have been carried out in other geographical areas and have shown similar results, namely that the prevalence of rotavirus antibody is high in newborns due to transfer of passive antibody from the mother, then diminishes in the first 6 months of life, is again very high by the age of 2–3 years, and is maintained throughout life. This pattern of rapid acquisition of antibody is comparable to that observed with respiratory syncytial and parainfluenza type 3 viruses.

The high prevalence of antibody in later childhood and adult life can be explained by the results of family studies from Canada, Norway, and the USA, in which up to 55% of older siblings and household contacts of paediatric patients with rotavirus gastroenteritis showed evidence of infection on the basis of serological studies. Most of the cases are asymptomatic and probably represent reinfection. It is not known whether it is the infected younger child or the usually asymptomatic adult member who is most likely to introduce rotavirus into the family unit.

Recent evidence has demonstrated the existence of more than one serotype of human rotavirus. Workers in Belgium using a CF assay and immune electron microscopy, in England using neutralization of immunofluorescent foci, and in the USA using ELISA have defined two distinct serotypes. These serotypes appear to be widely distributed geographically. In the Washington, DC, metropolitan area, the sera of most children aged 2 years contained antibodies to both serotypes, and in hospitalized patients type 2 rotavirus was seen more frequently. In addition, studies of patients who had experienced sequential infections revealed that illness caused by one serotype did not provide protection against illness caused by the other serotype. It is not certain whether other serotypes exist; workers in England using a fluorescent neutralization test have claimed to have found two other serotypes.

Seasonality

It has been clearly demonstrated in studies in Australia, England, and North America, that in temperate climates rotavirus disease is much more prevalent in the colder season. One exception may be infection in newborns; in Sydney, Australia, no seasonal variation

was found when rotavirus infection was studied in nurseries for the newborn. Whether this seasonal pattern occurs in developing countries with tropical climates is unclear. In studies from Costa Rica and Venezuela little or no seasonal variation in occurrence has been observed; however, in studies in Bangladesh and in Calicut and Vellore, India, rotaviruses have been found most frequently in stool samples collected from diarrhoea cases between November and March, which are the coolest months of the year. In the Vellore study, rotaviruses were present in the neonatal nursery throughout the year, as was observed in the Australian study cited above. In one small study conducted in Mexico City, where there is almost no seasonal difference in temperature, there was a peak of cases in the autumn months.

Transmission

All evidence to date indicates that rotavirus infection spreads by faecal–oral transmission; this has been confirmed by volunteer and animal experiments. There is no evidence to suggest that rotavirus multiplies with production of infectious particles anywhere else than in small bowel enterocytes.

Although IgA specific antibody has been found in the colostrum and breast milk of lactating mothers in a number of countries, it is not clear what role breast milk plays in protection against rotavirus disease, especially in developing countries where breast feeding frequently continues past the sixth month of life, when rotavirus disease is common.

Outbreaks of rotavirus diarrhoea have been documented in nurseries, especially those providing special care, and in a number of paediatric hospital wards. This problem of nosocomial transmission of rotavirus has been a difficult one to control.

Role of rotavirus in disease other than acute enteritis

Rotavirus infection has been described in children with intussusception and children with gastroenteritis and self-limited gastrointestinal bleeding. Clinical findings compatible with Henoch-Schoenlein purpura developed in one child in the latter group. In another report, a child with rotavirus-associated gastroenteritis had a severe central nervous system manifestation and developed fatal Reye's syndrome. In another child, encephalitis was reported. Several children with rotavirus infection have developed the haemolytic uraemic syndrome or disseminated intravascular coagulation. Elevated SGPT and SGOT levels have also been reported in some cases. In addition, there are reports that rotavirus has been isolated from intestinal tissue obtained from patients with Crohn's disease. One such report could not be confirmed in another laboratory, in which a contaminating, fastidious *Mycoplasma* was detected from such a patient. The isolation in cell culture of a rotavirus from extracts of mesenteric lymph nodes from a patient with Crohn's disease has been described recently. The role of rotavirus in the above conditions is unclear and needs further study.

Rotavirus diarrhoea in animals

Epidemiological features

Rotavirus has been shown to be a common intestinal infection in animals and has been demonstrated as a cause of diarrhoea in mice, calves, piglets, foals, young rabbits, deer, a pronghorn antelope, chickens, turkeys, goats, kittens, a chimpanzee, and a gorilla. There is also evidence that it can infect other animals, as shown by virus isolation studies in monkeys and dogs, and serological studies in goats, dogs, and guinea pigs. From virological and serological studies it is evident that infection occurs in early life in 90–100% of pigs and calves, as is the case in man, but at a lower level (38% in one study) in sheep. This lower infection rate in sheep may reflect the different systems of husbandry; the sheep studied

lived under less crowded conditions which may have reduced the probability of transmission. Laboratory colonies of rabbits and guinea pigs show a wide variation in the number of animals with antibody to rotavirus (0–100%). However, under natural conditions of husbandry, where populations have an urban distribution or where many young are congregated, the incidence of rotavirus infection in these species frequently reaches 100%.

In pigs and calves the severity of rotavirus infection, as judged by mortality, is usually less than that associated with *Escherichia coli* or coronavirus infections, although epizootic disease has had a reported mortality of up to 90%. However, it should be borne in mind that whereas *E. coli* and coronavirus infections can be diagnosed efficiently by staining of intestinal tissue obtained at autopsy, rotavirus infection cannot be so diagnosed and often requires electron microscopy for its detection. Some veterinarians have observed an apparent association of other agents, including bacteria and viruses, and also environmental stress with increasing severity of disease in naturally occurring epizootics.

The epidemiology of rotavirus infection in animals is not well understood. Although the disease usually occurs in infants or neonates, diarrhoea has been seen in adult animals in whom the rotavirus serum antibody titres are zero or low; often both the dam and her offspring have concurrent illness. It is probable that intestinal immunity of a level sufficient to prevent reinfection of the gut lasts not more than 6 months following infection, and thus the virus may be circulating constantly in the adolescent and adult population. In addition, the great stability of the rotavirus in faeces and its resistance to pH changes (pH 2–9.5), temperature (60°C), and a number of commonly used disinfectants, probably result in prolonged survival of the virus in contaminated buildings and water supplies.

It has been suggested that rotavirus may cross the placenta and infect the fetus, which would then be born either with infection or with actively acquired immunity. This hypothesis was based on reports, largely unpublished, that antibody to rotavirus was found in fetuses and that calves were frequently ill within 48 hours after birth. However, of the many hundreds of calves and pigs obtained by Caesarean section for rotavirus research from herds in which rotavirus infection was common, none has been shown to possess serum antibody, to be immune to experimental infection, or to be actively excreting virus. Since the incubation period for rotavirus infection is short (1–2 days), it is more probable that most infections in early life occur as a result of postpartum contact with an infected person or environment.

Immunological considerations

Preliminary data suggest that, although all rotaviruses have a common antigen, a wide range of antigenic subtypes can be differentiated by laboratory tests (see page 190). At present, these are recognized mainly among isolates obtained from different mammalian species, although at least two subtypes have been reported among human rotaviruses (see page 185). Most rotavirus strains tested under experimental conditions show cross-infection between mammalian species (e.g., human, bovine, equine, porcine, and ovine rotaviruses can infect piglets; human rotaviruses can infect monkeys; human and equine rotaviruses can infect calves). Some, but not all, of these cross-infections cause diarrhoea (see page 184). There has been no evidence to prove that rotaviruses cross species barriers under natural conditions. However, in England one human rotavirus isolated from a child was found to be serologically close to a bovine strain, and one isolate from a pig was serologically more closely related to a bovine than to a porcine strain. Thus it appears that different rotavirus strains exist with different antigenic specificities. Whether the different antigenic specifications would be likely to cause problems in a vaccination programme is not known. However, with a closely related virus, blue tongue of sheep, there are at least

22 strains which, although they share a common antigen, show relatively poor cross-protection. This virus infection is limited to sheep (virulent infection), to cattle (largely subclinical), and possibly to wild ungulates. In comparison, rotaviruses are more widely distributed in the animal kingdom and thus can be expected to show as wide, or wider antigenic variations.

Cross-protection studies between different animal species in different laboratories have given conflicting results. In one study, 3 gnotobiotic calves were inoculated with foal rotavirus and 3 with human rotavirus within 3 days after birth and none in either group developed a diarrhoeal illness; however, on challenge with bovine rotavirus 21 days after the initial inoculation, 2 of 3 animals in each group developed a diarrhoeal illness, suggesting that only limited, if any, protection was induced by the initial inoculum. In two more recent studies in pigs, bovine rotavirus and human rotavirus were ineffective in protecting piglets from later challenge with porcine virulent rotavirus. In another study of the relationship between human, ovine, equine, and porcine rotaviruses in pigs, all of which are separable by a neutralization test, there was poor evidence of cross-protection. In contrast to these studies, other workers have reported good evidence of cross-protection, including the demonstration that (a) bovine colostrum can protect pigs against challenge with a porcine isolate of rotavirus, (b) bovine rotavirus vaccine can induce protection against human type 2 rotavirus in calves, and (c) pigs infected with human rotavirus type 2 are protected against later challenge with porcine rotavirus. In none of these studies were the isolates used of the same origin, nor was the ID₅₀ of the challenge virus determined.

It is thus evident that further studies are required to determine the amount of cross-protection between rotavirus strains of different species. Even if cross-protection between strains is demonstrated experimentally, the efficiency of the protection may be less under field conditions as most reports of experimental studies indicate a lower mortality from rotavirus infections than that seen in natural outbreaks, except in newborn piglets.

In contrast to the conflicting data on cross-protection, there is good experimental evidence that animals can be effectively immunized against disease induced by rotavirus of their own species. Although limited, all the work published to date has shown that animals that are inoculated with rotavirus and allowed to recover are, on challenge 3 weeks later, immune to rotavirus illness and shedding. Similarly, calves vaccinated with an attenuated live rotavirus are immune to challenge with the parent virulent virus or with another bovine virulent rotavirus of close antigenic relationship. Also, virulent virus in the presence of colostral antibody in the intestine induces a subclinical infection that immunizes the animals against illness and rotavirus shedding for at least a limited period. It can be concluded from the available evidence that rotavirus infection in mammals results in the development of immunity to illness and rotavirus shedding, and it is most likely that for vaccines to be effective they will need to protect against each serotype that can infect the particular mammalian species under consideration.

Laboratory diagnosis

Methods for the detection of rotavirus in stools

In human infections large numbers of rotavirus particles are excreted in the stool. The optimal period for virus detection is within the first 3–5 days after onset of symptoms. The virus particles can usually be seen easily by negative-stain electron microscopy (EM) following differential centrifugation; in many instances they may be seen without centrifugation. The size and the characteristic double-capsid structure of the particles can be readily recognized. In stools that contain few virus particles the use of immune electron microscopy (IEM), i.e., the addition of specific antiserum to the stool extract to aggregate the particles, may occasionally make them easier to identify.

Use of electron microscopy is an efficient way of detecting rotavirus infection, but it is costly in time and equipment, and most developing countries do not have such facilities. Thus, other methods to detect virus particles have been explored.

One of these is immunoelectro-osmophoresis; this method is rapid and cheap but its sensitivity depends on the quality of the antiviral serum used. Although some workers find it more sensitive than EM, most have found it either as good or somewhat less sensitive.

The use of a special modified complement-fixation test (CF) to detect rotavirus in stool suspension has also been reported and has been found to be almost as sensitive as EM. One major problem is that many stool extracts are anti-complementary, although the addition of fetal calf serum may overcome this difficulty. With this method, high-titred human sera were found to be better than rabbit sera for the detection of human rotavirus.

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) have been used successfully for the detection of human and calf rotaviruses. The methods are claimed to be at least as sensitive as EM and probably more sensitive. With ELISA, the specificity of positive specimens has to be established with appropriate "blocking" reagents. The constraints with RIA are that sophisticated equipment is required and radioactive material may be hazardous. In this respect, ELISA shows more promise, especially because it can be used under field conditions to examine a large number of stool specimens.

Immunofluorescence (IF) has been used to stain cells or cell debris excreted in stools from infected calves. The problem of non-specific fluorescence has prevented its widespread use on human stools, but this can be overcome if the virus antigen is first separated by immune precipitation prior to staining. However, this method is laborious and time-consuming when done on a large scale. A simpler method is needed to make the use of IF more acceptable.

A new test termed solid-phase aggregation of coated erythrocytes (SPACE) has been described, which combines some of the features of ELISA and of solid-phase radioimmunoassay. This method involves the coating of microtitration U-plates with specific antiviral antibody and the addition of 10% faecal suspensions. The absorbed viral antigens are then detected by the addition of erythrocytes coated with specific antiviral IgG. The results are read in the same manner as the conventional haemagglutination test. This test has been shown to give very good agreement with both EM and IF, but appears to be limited in application because of the need for frequent preparation of coated erythrocytes.

As mentioned previously, one of the most urgent needs is to find a simple method for the efficient propagation of human rotavirus in cell culture; this would undoubtedly also improve diagnostic tests. Enhancement of virus growth in cell cultures by adding proteolytic enzymes to the culture medium has been reported. A cell culture method that is capable of demonstrating the presence of human rotavirus antigen, usually in a single passage, has been employed successfully; this involves centrifugation of stool specimens on to a guinea-pig kidney cell line followed by IF staining. This is a relatively simple way of diagnosing rotavirus infection, titrating virus infectivity, and estimating neutralizing antibodies in serum. Serial propagation of human rotavirus by this technique alone has not been reported.

Methods for the measurement of rotavirus antibody

A wide variety of tests have been developed for the measurement of rotavirus antibody. IEM was used in some earlier studies on human sera; however, for general serological surveys or for serodiagnosis a simpler and quicker method was needed and the complement-fixation test (CF) was shown to meet this requirement. Initially the CF antigen used was human rotavirus obtained from stool extracts, but it was found that this could be replaced by the more readily available bovine rotavirus grown in cell culture, or even more efficiently by the O agent (see page 183). One limitation of the CF test is that it does not

distinguish between antibodies of different species origin. Another simple and cheap method for detection of group-specific rotavirus antibody is radial immunodiffusion (ID). With ultracentrifuged concentrates of stools as antigen, a single strong line common to all the rotaviruses can be seen against antisera from convalescent children and animals. The IF test has been used to measure serum antibody titres against human rotavirus. Antibodies have also been quantitatively detected by RIA and ELISA using the "double-antibody sandwich" technique. These tests are very sensitive and have the added advantage of being adaptable to measure specific immunoglobulin classes of antibody. Anti-rotaviral IgA has also been measured by IF.

Methods for the identification of rotavirus strains and serotypes

CF, IF, ID, and IEM techniques have shown that rotaviruses share a common group antigen that is associated with the inner capsid of the virus particle. However, human and animal rotaviruses can be distinguished by neutralization of immunofluorescent foci and ELISA blocking techniques. A one-way serological relationship between human and simian rotaviruses has been reported as a result of IEM studies; a one-way ELISA blocking test showed these viruses to be distinct.

The existence of two rotavirus serotypes in man has been demonstrated by neutralization tests, CF, and ELISA. Two additional serotypes have been described by workers using the neutralization technique (see page 185).

Electrophoretic migration of viral nucleic acid has been useful for distinguishing viruses and is based on differences in the migration of RNA segments on polyacrylamide gels. RNA analyses can be carried out directly on most stool samples in which rotavirus particles are visible by EM. However, a correlation between serotypes and "electrophoretotypes" has not been established and a difference detected by this technique may not necessarily reflect an antigenic difference.

DIARRHOEA DUE TO NORWALK AND NORWALK-LIKE AGENTS

Description and characteristics of the Norwalk-like agents

Since 1972, a new group of agents, of which the 27-nm Norwalk particle is a prototype, has been discovered and found to be associated with outbreaks of generally mild gastroenteritis occurring in school, community, and family settings. In antibody prevalence studies employing a newly developed immune-adherence haemagglutination assay (IAHA), it has been found, in the Washington, DC, metropolitan area, that antibody to the Norwalk agent is acquired gradually in childhood and more rapidly in adult life, so that antibody is present in 50% of the population in the fifth decade of life. This pattern of antibody acquisition contrasts markedly with that of rotavirus infection and suggests that the Norwalk agent and probably other morphologically similar agents are not a major cause of gastroenteritis in infants and young children but are more likely to be primarily associated with illness in older children and adults.

The Norwalk agent was initially discovered in an outbreak of gastroenteritis that occurred in Norwalk, Ohio, in which 50% of the pupils and teachers in an elementary school and 32% of the family contacts became ill. A filtrate made from a rectal swab obtained from a secondary case was administered orally to three adult volunteers and

produced disease in two; it was subsequently successfully passaged serially. The agent could not be propagated conclusively in any cell or organ culture system and was first visualized by immune electron microscopy (IEM) in an inoculated volunteer's infectious stool filtrate. It has recently also been detected in vomitus from 4 of 5 volunteers who developed gastroenteritis after administration of a 2% filtrate of the agent.

Particles resembling the Norwalk agent morphologically and in density were subsequently discovered by IEM in individuals in two family outbreaks of gastroenteritis, one in Hawaii (Hawaii agent) and the other in Montgomery County, Maryland (MC agent). By IEM and cross-challenge studies, the Hawaii and Norwalk agents were found to be distinct, the Norwalk and MC agents to be related, and the relationship between the Hawaii and MC agents was inconclusive. Viruses resembling the Norwalk agent in morphology and density were later observed in faecal specimens from: (a) a gastroenteritis outbreak in a primary school in Ditchling, England; (b) a volunteer who became ill after administration of the "W" agent that had been obtained from a boy who developed gastroenteritis during an outbreak in a boarding school; and (c) individuals developing gastroenteritis after eating cockles. By IEM, the Ditchling and "W" agents are related but are distinct from the Hawaii and Norwalk agents, whereas the cockle agent appears to be distinct from the Norwalk agent. However, the relationship of the cockle agent to the other agents is inconclusive. Thus, besides the cockle agent, there appear at present to be three serotypes of this group of agents.

These agents have a diameter (shortest diameter) of 25–27 nm. They are not perfectly round, the Norwalk agent, for example, averaging 27 nm in its shortest diameter and 32 nm in its longest; they are similar morphologically to the picorna or parvoviruses and the hepatitis A virus. A clear-cut substructure is not visible by electron microscopy (EM). They have not yet been propagated definitively *in vitro* and therefore must be detected by EM, IEM or, in addition, for the Norwalk agent, by the recently developed radioimmunoassay (RIA) or IAHA.

These agents have a buoyant density in caesium chloride of 1.37–1.41 kg/litre as determined by ultracentrifugation and IEM or EM. Their nucleic acid and protein contents are unknown. The Norwalk agent is stable after exposure to pH 2.7 for 3 hours at room temperature, as determined from infectivity studies in volunteers. Similar studies have shown that the Norwalk and "W" agents are stable after exposure to 20% ether at 4°C for 24 and 18 hours, respectively. The Norwalk agent is relatively heat-stable, retaining infectivity in volunteers after heating at 60°C for 30 minutes.

These agents have not yet been classified. However, since the Norwalk agent shares certain characteristics with the parvoviruses, such as morphology, density, and ether, acid, and relative heat resistance, it appears to be parvovirus-like. It should be emphasized however, that none of this group of agents has had its nucleic acid content identified; thus they cannot be classified into any group.

The Norwalk agent has been administered to mice, guinea pigs, rabbits, kittens, calves, baboons, chimpanzees, and rhesus, marmoset, owl, patas, and cebus monkeys; none of the animals developed illness. When paired sera from monkeys, baboons, and chimpanzees were examined for a Norwalk serological response, only the chimpanzee developed such a response. Chimpanzees were also found to be shedding Norwalk antigen when examined by RIA after challenge.

Clinical and epidemiological characteristics

Of the 604 primary and secondary cases in the original Norwalk outbreak, 85% had nausea, 84% vomiting, 52% abdominal cramps, 57% lethargy, 44% diarrhoea, 32% fever,

and 5% chills. Symptoms lasted 12–24 hours in the majority of cases and seldom longer than 48 hours; none of the patients was hospitalized. The average incubation period was 48 hours. The signs and symptoms of illness observed in 32 of 55 volunteers who developed illness following the administration of 2% filtrates of stool containing Norwalk agent were very similar to those observed under natural conditions: incubation period 10–51 hours, fever (37.4°C) in 16 (50%), diarrhoea in 27 (84%), vomiting in 20 (63%), abdominal discomfort in 23 (72%), anorexia in 30 (94%), headache in 27 (84%), and myalgias or malaise in 20 (63%). The clinical manifestations usually lasted for 24–48 hours. The illness was generally mild and self-limited, although a volunteer who vomited approximately 20 times within a 24-hour period required parenteral administration of fluid. Shedding of the Norwalk particle in volunteers, as determined by IEM, was at a maximum during the first 72 hours after onset of clinical illness and occurred only infrequently afterwards. The signs and symptoms of illness observed in volunteers following administration of the Hawaii, MC, and “W” agents have been similar to those observed with the Norwalk agent.

In volunteer studies with the Norwalk agent short-term and long-term immunity have been observed. It appears that mechanisms other than that mediated by serum antibody are of primary importance in immunity to Norwalk gastroenteritis.

In volunteers challenged with the Norwalk or Hawaii agents, characteristically, there is broadening and blunting of the villi of the jejunal mucosa; the mucosa itself is histologically intact. Moderate amounts of mononuclear cell infiltrates and cytoplasmic vacuolization of epithelial cells have also been observed. Examination of thin sections of the jejunal mucosa by transmission electron microscopy has revealed intact epithelial cells with shortening of microvilli, dilatation of rough and smooth endoplasmic reticulum, swelling of mitochondria, and an increase in lysozymes; virus particles were not seen inside these cells. Biopsies taken 6 or more weeks after infection have been normal. During Norwalk illness, the gastric mucosa appeared normal histologically. Small intestinal brush-border enzymes (including alkaline phosphatase, sucrase, and trehalase) were decreased; significantly, adenylate cyclase activity in the jejunal mucosa during Norwalk or Hawaii illness was found not to be elevated.

The role of the Norwalk agent in outbreaks of gastroenteritis has recently been investigated employing the newly developed Norwalk RIA and radioimmuno-blocking assay. Six of 23 outbreaks were found to be associated with the Norwalk agent; of these, two occurred on college campuses in the USA, one in a primary school in Japan, two on cruise ships, and one in two members of a family, one of whom was involved in an elementary school outbreak while the other, a secondary case, developed a serological response and shed a morphologically Norwalk-like particle. If one includes the original Norwalk outbreak as well as the serologically related outbreak in Montgomery County, both of which were associated with Norwalk-like particles as determined by IEM, 8 separate outbreaks of gastroenteritis (32%) have been identified among 25 studied. If suitable serological tests had been available for Hawaii and “W” agents, it is possible that the majority of the outbreaks would have had an assignable etiology. In addition, a recent study in Australia suggests that at least part of another outbreak of gastroenteritis related to the eating of oysters was associated with the Norwalk agent.

Laboratory diagnosis

Methods for direct detection in stool of Norwalk and Norwalk-like agents

Immune electron microscopy (IEM) was used for the initial detection of the Norwalk agent. To detect the Norwalk agent in stool material, a stool suspension or filtrate is

incubated with a convalescent Norwalk serum or immunoglobulin. The mixture is then centrifuged, the supernatant poured off, and the pellet or sediment reconstituted with distilled water and examined with a negative stain by electron microscopy.

The Norwalk group of agents cannot be identified directly by electron microscopy (EM) because they do not have a sufficiently distinct morphology. However, the Ditchling and "W" (and cockle) agents have been examined by EM after concentration by ultracentrifugation, followed by density gradient centrifugation in caesium chloride: for EM, a drop of fluid from a fraction was placed on a slide covered with 0.9% agarose, a grid was subsequently placed on the drop for 30 minutes to permit the caesium chloride to diffuse into the agarose, before examining the grid with a negative stain.

A radioimmunoassay (RIA) has recently been developed for detection of Norwalk agent in faecal specimens and appears to be even more efficient than IEM. The assay is based on a differential binding of stool suspensions containing Norwalk particles to a microtitration well coated with convalescent and another coated with preinfection anti-Norwalk antibody. By this method, non-specific interaction can be separated from specific Norwalk binding. The assay can detect soluble viral antigen as well as particulate material. Unfortunately, some stool specimens (less than 5%) have high non-specific activity that masks specific Norwalk binding. The success of this technique is dependent on the availability of specific reagents, and the only available, satisfactory antisera to the Norwalk agent have been obtained from human subjects or chimpanzees infected with the Norwalk agent. High-titred convalescent and antibody-negative preinfection sera from the same person or chimpanzee must be used. At present, these sera are not available commercially.

Methods for measurement of antibody to Norwalk and Norwalk-like agents

Until recently IEM was the only method for detecting a serological response to the Norwalk agent. Since IEM is employed for the detection and recognition of the Norwalk agent, an IEM serological assay with appropriate paired sera is essential to determine the significance of a particle visualized as a result of reaction with convalescent serum or immune serum globulin.

More recently, an immune adherence haemagglutination assay (IAHA) has been developed. In this technique, the Norwalk antigen is prepared from human stool containing the Norwalk particle using various extractions and concentration procedures. The test employs several reagents and human O erythrocytes, and has similarities to both a complement-fixation (CF) and a haemagglutination test. It has been quite specific for detecting serological responses as it has a high degree of concordance with IEM and RIA-BL (see below), but IEM and RIA-BL are slightly more efficient. In addition, IAHA cannot be used to detect Norwalk antigen in stool specimens. A major drawback with the use of IAHA for serological studies is the lack of an adequate supply of Norwalk antigen. Although IAHA requires considerably less antigen than CF, it still uses considerably more than the RIA-BL test. In addition, not all human O erythrocytes react comparably in the IAHA and it may be necessary to screen numerous donors' erythrocytes to find a donor that reacts with enough sensitivity to a known antigen.

The radioimmunoassay for detecting Norwalk antigen has been modified to identify Norwalk antibody in the form of a radioimmunoassay blocking test (RIA-BL). The assay is based on the ability of a test serum to block the binding of ^{125}I IgG anti-Norwalk to Norwalk antigen; it is highly sensitive and specific and was found to be slightly more efficient than IEM in detecting serological responses in volunteers challenged with Norwalk agent. The RIA-BL test is suitable for large-scale studies and can theoretically be used to detect antibody in body fluids other than serum (i.e., intestinal secretions or milk).

OTHER VIRAL DIARRHOEAS

A number of viral agents have been identified whose role as etiological agents of acute diarrhoea is not clear. These can be divided into two groups. One includes those viruses such as adenoviruses, coronaviruses, and enteroviruses that are universally recognized as mammalian viruses; the other includes particles or agents that have been described by a number of investigators and termed astrovirus, calicivirus, and mini-rotavirus. The available knowledge on these agents is summarized below.

Adenovirus

Adenoviruses are well established as respiratory viruses (33 serotypes have been recognized in cell culture) and the common serotypes can be isolated from stools in cell culture. Recently, a number of workers have reported visualizing by electron microscopy (EM) morphologically indistinguishable viruses in stool which could not be grown subsequently in cell culture. The reason for this phenomenon is unknown, but it has prevented more than tentative identification of such strains. Adenoviruses have been isolated from many animal species but none has been clearly implicated as a cause of diarrhoea.

In several studies adenoviruses have been found by EM in stools from 5–8% of normal children; in some, prolonged excretion was common. No one serotype has been associated convincingly with diarrhoea. In one recent outbreak the virus could be identified only by immune electron microscopy (IEM) and could not be grown. The role of adenoviruses, especially those that do not grow or grow poorly in cell cultures, as etiological agents of diarrhoea needs further study. In volunteer studies, which have been confined to well established strains, viruses were grown from faeces but gastrointestinal symptoms were not seen.

Astrovirus

Astrovirus was first described in Scotland in 1975 in the diarrhoeal faeces of infants examined by EM and has subsequently been observed elsewhere in the United Kingdom. Very similar, but possibly not identical, particles have been reported from Australia and Canada. These particles cannot be grown in routine cell cultures but limited growth has occurred in human embryo kidney (HEK) cells; growth was detected by immunofluorescence using an antiserum prepared in guinea pigs. No evidence has yet been found of the existence of more than one serotype.

The role of astrovirus as an etiological agent is unclear. In one study, they were found more frequently in the stools of infants with diarrhoea than in controls; they have also been implicated in two outbreaks in the United Kingdom. Postinfection increases in antibody have been detected by IEM. In adult volunteers challenged with astrovirus, infection was common and was accompanied by excretion of large amounts of virus and by seroconversion, but illness occurred in only a small number of individuals.

Morphologically similar viruses have been found in the faeces of lambs and calves. The lamb virus has been shown to cause diarrhoea in gnotobiotic lambs, but the calf virus induced only seroconversion in gnotobiotic calves. Astroviruses from man, lambs, and calves have been reported to be serologically distinct.

Calicivirus

This agent was first described in a community survey in Scotland in 1976, where it was detected by EM in the faeces of infants, and similar viruses have been reported by two other

laboratories in the United Kingdom. Apparently similar particles have been reported by two laboratories in Canada, but in both cases they were labelled astroviruses and thus the situation is somewhat confused. No growth of this agent in cell cultures of human or feline origin has been reported, and different serotypes have not been described.

In one report, calicivirus particles were detected in the faeces of children involved in a school outbreak of "winter vomiting". The particle was found only in the faeces of patients and not in contacts. One adult teacher was also affected, but did not seroconvert. Other reports have indicated a less definite association between calicivirus and gastroenteritis. No volunteer studies have been reported. Morphologically similar viruses have been isolated from pigs, cats, and sea-lions, though none of these agents has been shown to cause diarrhoea in the natural host. It is not yet clear whether there is more than one serotype of the feline virus.

Coronavirus

The importance of coronavirus as a cause of diarrhoea remains unknown. In one report coronaviruses were detected by EM in stools obtained from 3 outbreaks of gastroenteritis in adults and the particles in a stool from one of the outbreaks were propagated in organ and cell cultures. No volunteer studies with the virus have been done. Coronaviruses, however, naturally infect many animals (chickens, mice, pigs, calves, dogs, rats, and cats) in which they cause a variety of clinical symptoms including diarrhoea in certain animals.

Enterovirus

Sixty-eight enteroviruses have been identified, all of which, with the possible exception of 2, can be isolated from faeces. Electron microscopy showed them to be small (25-nm) featureless spheres indistinguishable from one another. Techniques for isolating enteroviruses have been available for over 10 years and numerous investigations have attempted to implicate them as causes of diarrhoea. In general, no convincing evidence has accrued, though from time to time outbreaks apparently involving well-characterized types have been reported (for example, echovirus types 11, 14, 18, and 19). Whether such outbreaks were due to the enterovirus or to an undetected agent is not known. There is no evidence that the well known pathogenic enteroviruses like the polioviruses and coxsackieviruses cause diarrhoea.

The absence of any clear association between enteroviruses and diarrhoea has stimulated few volunteer studies. Some studies have been performed using strains of echoviruses and coxsackie A viruses isolated from the throat, but these caused little or no gastrointestinal illness.

Enteroviruses are found in a number of animal species but there is no evidence that they cause diarrhoea in their natural host.

Other small, round, virus-like objects (SRVs)

Some stool extracts have been shown to contain other virus-like particles 25–35 nm in diameter which to some investigators are clearly different from the background materials; they have not been grown in cell cultures. These SRVs have been reported mostly in association with outbreaks of gastroenteritis in communities or institutions. Within each outbreak the SRVs were consistent in size and appearance, but they differed between one outbreak and another. Several investigators have reported SRVs in the faeces of patients with sporadic diarrhoea (e.g., the "mini-rotaviruses" described in Canada, which were

morphologically similar to the viruses observed in an outbreak in Scotland) and in the absence of associated outbreaks it has been difficult to obtain enough material for their detailed investigation. Similar SRVs have been seen in the faeces of dogs and pigs with diarrhoea, but their relationship to human SRVs, if any, is unknown.

RECOMMENDATIONS FOR RESEARCH

Rotaviruses have emerged as the most important viral agents associated etiologically with severe diarrhoeal disease in infants and young children, and simple techniques are available for their identification. Therefore, priority should be given to research aimed at elucidating the epidemiology, pathophysiology, and means of prevention of illnesses caused by rotaviruses. Norwalk and Norwalk-like agents have also been recognized as a cause of diarrhoeal diseases, predominantly in older children and adults.

The following recommendations are made for further research:

Rotavirus diarrhoea

1. Studies are needed to define more completely the mortality and morbidity attributable to rotavirus diarrhoea in different geographical areas. Any factors, either host or environmental, that affect the severity of the disease need to be identified.

2. The epidemiological characteristics of rotavirus diarrhoea in different geographical areas also need elucidation. Studies should seek to define the following: relative incidence of the two recognized serotypes; seasonal occurrence of the disease; its modes of transmission; natural history of the disease; reinfection rate; incidence of asymptomatic infection; the relationship between nutritional status and the incidence of disease; possible association of the virus with chronic diarrhoea; the natural reservoir of human rotaviruses; factors influencing the survival of rotaviruses in the environment and the physicochemical stability of the virus.

3. Studies are needed to determine why human neonates infected with rotavirus often experience mild disease or subclinical infection. The relative infrequency of severe disease in human neonates with rotavirus infection is in direct contrast to the natural history of such infection in older infants and in young children; in the animal world also, the newborn experience the most severe rotavirus illness. These studies should consider such factors as immune status, virulence of the virus, diet, and influence of other faecal organisms. Information is also needed to learn whether human neonates with initial subclinical infection are susceptible to reinfection and/or disease from the same or another serotype later in life, or whether they are rendered immune by the initial infection.

4. Further studies are needed to determine the pathophysiology of rotavirus diarrhoea.

5. The effect of feeding during diarrhoea on the clinical course of rotavirus diarrhoea needs to be determined.

6. The role, if any, of rotavirus disease in malabsorption and subsequent nutritional deficiencies should be ascertained.

7. More research should be undertaken on the serotyping of rotaviruses isolated from animals and humans, to better define possible reservoirs of infection. Under experimental conditions, rotavirus virulent for one mammalian species may infect another species subclinically. Thus, under natural conditions, it is possible that human contacts could represent a source of infection for animals and that animal contacts are a source of infection for humans.

8. Research is needed to find methods for determining the virulence of rotavirus in animals and humans, both as a means of comparing different isolates and also as a method for determining whether a causal relationship exists between a virus present in the faeces and the clinical syndrome.

9. Since human rotaviruses do not grow to sufficiently high titre to allow them to be used directly in a vaccine development effort, a high priority should be given to studies of methods for more efficient propagation of these agents.

10. Although intestinal IgA rotavirus antibody is known to be of prime importance in preventing rotavirus illness, additional studies are needed to establish the duration of, and means of enhancing, the immune response. Studies of rotavirus antibody levels in secretions such as saliva and breast milk are required to determine whether they reflect antibody content in small-intestinal fluid.

11. Studies should be carried out to determine the influence of breast feeding on the natural history of rotavirus infection. Epidemiological, immunological, and social factors should be investigated. Knowledge gained from such studies would help to determine whether a rotavirus vaccine should be administered to women of child-bearing age.

12. Since passive administration of rotavirus antibody by the alimentary route has resulted in resistance to rotavirus challenge in various animal models, studies in humans on the effect of oral administration of human rotavirus antibody might be considered. Another approach might be the oral administration of purified separated fractions of cow's "immune milk" (containing antibody to rotavirus).

13. An experimental animal in which disease could be induced beyond the early period of life should be sought. This is important for studying the safety and efficacy of candidate rotavirus vaccines and for studies of virulence.

14. The group recognizes the need to support studies aiming at the development of a rotavirus vaccine. In that regard it endorses the recommendations of the Scientific Working Group on Immunity and Vaccine Development.^b

15. There is a need for further development and evaluation of simple and reliable techniques for the detection of rotavirus and for the identification of serotypes.

16. In order to conduct most of these studies, a supply of high-quality reference reagents must be available. WHO should immediately assume the preparation and distribution of the following standard reagents:

(a) Pre-immunization and hyperimmune serum to rotavirus, prepared in goats and guinea pigs.

(b) Pre-infection and convalescent (3–4 weeks) antiserum to rotavirus prepared in gnotobiotic calves.

(c) Rotavirus antigen for use in ELISA and other immunological tests. To standardize diagnostic tests worldwide, a single antigen should be used in the preparation of (a) and (b). Such a reagent is in the process of preparation under contract to the National Institutes of Health, USA, and the Pan American Health Organization, and every effort should be made to distribute it globally as a WHO reference reagent. At a later date, when techniques are available, specific hyperimmune antiserum to the two human rotavirus serotypes should be developed and made available through WHO.

17. Training materials should be developed and workshops conducted for training laboratory workers to perform diagnostic tests.

Diarrhoea due to Norwalk and Norwalk-like agents

1. Since the Norwalk group of agents do not grow in a cell culture system, efforts should be made to find a way of propagating these agents.

^b See *Bulletin of the World Health Organization*, 57 (5): 719-734 (1979).

2. Attempts should be made to find additional, serologically distinct 27 nm-like agents associated with viral gastroenteritis.

3. Practical detection methods and serological assays for other Norwalk-like agents, such as Hawaii, "W" or Ditchling agents, have to be developed so that their natural history can be further elucidated.

4. Additional efforts should be made to discover animal models for the study of illness caused by the Norwalk group of agents.

5. The observed absence of long-term immunity to Norwalk agent in one group of volunteers in contrast to another which had such immunity has raised important questions in connection with intestinal immunity. The reasons for this lack of immunity in one group need elucidation.

Other viral diarrhoeas

Research should be carried out to determine the significance of astroviruses, caliciviruses, coronaviruses, enteric adenoviruses (untyped), and other virus-like particles detected in stools from acute cases of human diarrhoea. This should include longitudinal and case-control epidemiological studies, and laboratory studies to find a way to propagate them *in vitro* for their further identification. Such studies should include an exchange of specimens between laboratories in order to agree on the identification of these different morphological entities.

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